

Resistance to Activation-Induced Cell Death and Bystander Cytotoxicity Via the Fas/Fas Ligand Pathway Are Implicated in the Pathogenesis of Cutaneous T Cell Lymphomas

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By engaging Fas, Fas ligand (FasL) on activated T lymphocytes induces activation-induced cell death (AICD), and also triggers apoptosis of target cells during immune downregulation. We previously showed that within cutaneous T cell lymphoma (CTCL) lesions, malignant CD4+ T cells expressing FasL accumulated, and were inversely distributed with CD8+ T cells. We thus determined the responses of human CTCL cells to AICD and their cytotoxic to Fas+ target T cells *in vitro*. CTCL cells expressing Fas were resistant to AICD following activation by CD3 monoclonal antibody (mAb) whereas still undergoing apoptosis if Fas was ligated to Fas mAb. CTCL cell lines, as well as Sézary Syndrome patients' peripheral blood lymphocytes, exhibited ratio-dependent cytotoxicity to Fas+ Jurkat cells. The kinetic study showed that FasL surface expression was absent before activation, and its expression was low and/or delayed after activation. We therefore hypothesize that CTCL cells express functional FasL possibly contributing to bystander cytotoxicity within tumor infiltrates. In addition, decreased and/or delayed FasL surface expression following activation may in part contribute to their resistance to AICD. Both bystander cytotoxicity and resistance to AICD are likely to contribute to the loss of cytotoxic anti-tumor CD8+ T cells as well as the accumulation of malignant T cells in CTCL.

Key words: activation-induced cell death/apoptosis/cutaneous T cell lymphoma/Fas/Fas ligand
J Invest Dermatol 124:741–750, 2005

Cutaneous T cell lymphomas (CTCL) are the most frequent extra-nodal lymphomas occurring in the skin (Weinstock and Horm, 1988). Mycosis fungoides (MF), and its leukemic variant, Sézary syndrome (SS), the most common CTCL variants, have been thought to arise from the accumulation and clonal expansion of skin homing, immunologically mature, CD4+/CD45RO+ (helper/memory) T lymphocytes (Tan *et al*, 1974). Persistent antigen stimulation, provided by skin organisms such as viruses and staphylococci, or by chemicals, has long been hypothesized to serve as the triggers initiating MF (Tan *et al*, 1974; Tokura *et al*, 1994; Jackow *et al*, 1997; Herne *et al*, 2003; Talpur and Duvic, 2003). Disease progress is characterized by increasingly impaired immunity with a shift to type 2 cytokine profile T-helper (Th)2, which is hypothesized to result from malignant T cell accumulation as well as loss of CD8+ tumor-infiltrating, cytotoxic T lymphocytes (Vowels *et al*, 1992; Heald *et al*, 1994).

Fas ligand (FasL), a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family, and its receptor, Fas (Apo-1/CD95), play an essential role in T cell

homeostasis (Nagata and Golstein, 1995; Bossi and Griffiths, 1999). FasL was described as a cytotoxic protein expressed on activated T cells (EL-Khatib *et al*, 1995). The primary functions attributed to FasL-mediated cytotoxicity are the ability of T cells to kill themselves during activation-induced cell death (AICD) and the ability of T cells to kill target cells in a calcium-independent manner (Lowin *et al*, 1996). In AICD-sensitive T cells, FasL expression is upregulated upon activation, and is delivered to the cell surface. FasL can then engage Fas on the same (suicide or AICD) or on a neighboring cell (patricide or bystander cytotoxicity) (Suda *et al*, 1995; Bossi and Griffiths, 1999; Green *et al*, 2003). CTCL cells are populations of activated mature T cells, which have prolonged life spans, and thus an abnormal cell death; specifically, a defective Fas/FasL pathway may result in accumulation of CTCL cells (Meech *et al*, 2001). Decreased Fas expression was found on peripheral blood CD4+ T lymphocytes in MF and SS (Dereure *et al*, 2000) and absent Fas expression was associated with more aggressive CTCL (Zoi-Toli *et al*, 2000). Because mutations of the Fas gene were detected in only 14% of CTCL patients' tumor cells (Dereure *et al*, 2002), other abnormalities linked to Fas/FasL signaling must exist in the pathogenesis of MF and SS.

Besides its presence on T cells, FasL is expressed on many other cells from different organs. Expression of FasL in the eye, placenta, and testis is suggestive of mechanism for immune privilege (Griffith *et al*, 1995). FasL can induce

Abbreviations: AICD, activation-induced cell death; CTCL, cutaneous T cell lymphoma; FasL, Fas ligand; FITC, fluorescein isothiocyanate; IL, interleukin; mAb, monoclonal antibody; MF, mycosis fungoides; MMP, matrix metalloproteinases; PBL, peripheral blood lymphocytes; PI, propidium iodide; SS, Sézary syndrome; Th, T helper

immune cells to undergo apoptosis, and thereby, limit the immune response in these organs. Tumor cells expressing FasL have been proposed to “counterattack” against activated anti-tumor effector immune cells, although some authors have indicated that FasL is not expressed on the surface of some solid tumor cells, such as colon cancer cells (O’Connell *et al*, 1996; Favre-Felix *et al*, 2000). We have previously shown that within CTCL lesion MF tumor cells highly express FasL, and were inversely distributed with CD8⁺ T cells (Ni *et al*, 2001); however, the direct evidence of FasL-mediated bystander cytotoxicity and cell surface FasL expression on CTCL cells has not been previously studied.

To better understand the mechanism of abnormal Fas/FasL pathway in both accumulation of neoplastic T cells and loss of immune cytotoxic CD8⁺ T cells in CTCL, we examined the susceptibility of human CTCL cells to AICD through ligation of the CD3/T cell receptor (TCR) as well as their ability to kill Fas⁺ T cell targets. We further assessed the induction of apoptosis following anti-Fas antibody treatment and the kinetics of FasL cell surface expression after activation. Although CTCL cells kill target cells via Fas/FasL pathway, they were less susceptible to AICD induced by anti-CD3 monoclonal antibody (mAb) than to anti-Fas mAb-induced apoptosis. This difference may be related to decreased or/and delayed FasL surface expression. These findings describe a novel mechanism that neoplastic T cells may utilize to resist AICD and thus accumulate in skin lesions.

Results

Resistance to AICD is not correlated with the level of Fas surface expression AICD is an apoptotic pathway involved in the control of T cell homeostasis (Lenardo, 1997). We therefore determined the susceptibility to AICD in response to CD3/TCR ligation in three CTCL cell lines. Compared with Jurkat cells, HuT78, MJ, and HH cells showed much less induction of apoptosis after CD3 mAb (2.5 μ g per mL) activation for 16 h. CD3 mAb activation induced an average 45.1% activation-induced apoptosis of Jurkat cells, but only 18.2% of HH cells, 6.1% of MJ cells, and none of HuT78 cells (Fig 1a).

Because AICD is primarily an Fas/FasL-mediated apoptosis, we next examined the Fas surface expression by flow cytometry using the ZB4 mAb. Surprisingly, Fas cell surface expression was present in all three tested CTCL cell lines (HuT78 = 94.6%; MJ = 97.7%; HH = 86.2%) (Fig 2a, b). Fas was also expressed on a high percentage (52%–80%) of CD4⁺ cells from four Sézary patients with high percentages of peripheral Sézary cells (Table I; Fig 2c).

Moreover, as shown in Fig 3, CTCL cells displayed concentration-dependent induction of apoptosis when they were treated with the apoptosis-inducing CH11 Fas mAb, which mimics the function of physiologic FasL, at different concentrations (0, 50, and 250 ng per mL). The sensitivity to CH11 Fas mAb among three CTCL cells was variable. HH cells showed highest sensitivity, with 41.5% and 54.0% apoptosis induction at 50 and 250 ng per mL Fas mAb treatment, respectively, whereas MJ cells just had 2.5% and

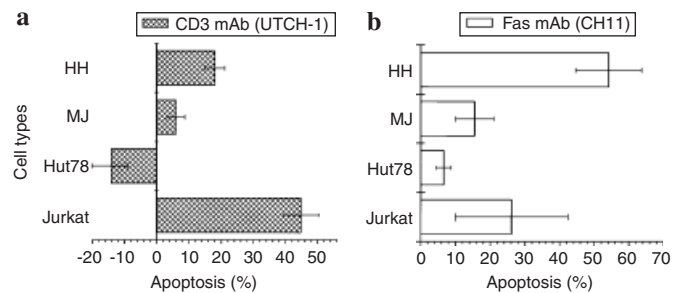


Figure 1

Cutaneous T cell lymphoma cells are resistant to activation-induced cell death activated by CD3 monoclonal antibody (mAb) whereas variably responsive to apoptosis-inducing Fas mAb. HuT78, MJ, HH, and Jurkat cells were activated on 2.5 μ g per mL UCHT-1 CD3 mAb-coated plates (a) or treated with 250 ng per mL CH11 Fas mAb (b) for 16 h. Apoptosis was measured by staining with fluorescein isothiocyanate-labeled AnnexinV and propidium iodide analyzed with a FACScan (Becton Dickinson).

15.5%, and HuT78 cells only with 3.6% and 6.6% (Fig 3). Compared with CD3 mAb activation, three CTCL cell lines were more sensitive to the treatment of CH11 Fas mAb (250 ng per mL) (Fig 1). Similar to HuT78 cells, all tested SS-peripheral blood lymphocytes (PBL) displayed resistance to both CH11 Fas mAb treatment and CD3 mAb-induced AICD as summarized in Table II. These results suggest that CTCL cells, especially HuT78 cells and SS-PBL, are resistant to CD3 mAb-induced AICD although Fas receptor is present on their surfaces with variable responses to CH11 Fas mAb treatment.

FasL surface expression is absent on CTCL cells at baseline Because cell surface FasL expression on activated T cells is necessary for inducing AICD of Fas⁺ cells (Bossi and Griffiths, 1999), using NOK-1 FasL mAb, specific for cell surface FasL, we next examined FasL expression on the surface of three CTCL cell lines (HuT78, MJ, and HH) and PBL from SS patients. As shown in Fig 4, flow cytometry (Fig 4a) and confocal microscopy (Fig 4b) showed that although FasL protein is easily detected on the surface of control KFL9 cells, surface FasL protein was absent on three CTCL cell lines (Fig 4a, b), as well as on all tested SS-PBL at baseline (data not shown).

FasL surface expression is decreased or/and delayed on CTCL cells after activation Hematopoietic cells store FasL in secretory lysosomes, which then undergo a polarized delivery to the interface between the effector cell and its target after activation (Suda *et al*, 1995; Bossi and Griffiths, 1999). This delivery of surface FasL occurs within minutes after activation, reaching the maximum level within 1–2 h with CD3 antibody activation or within 30 min with phorbol 12-myristate 13-acetate (PMA)/ionomycin activation (Nguyen and Russell, 2001). In order to study the kinetics of cell surface expression of FasL on CTCL cells, FasL surface expression was determined after activation with PMA (50 ng per mL)/ionomycin (1 μ g per mL) or CD3 mAb (2.5 μ g per mL) for 5, 30 min, 3, 16, 24, 48, and 72 h. Interestingly, cell surface FasL was undetectable after activation with either PMA/ionomycin or CD3 mAb for 5, 30 min, 3 and 16 h (data not shown) until 24 h later on HuT78

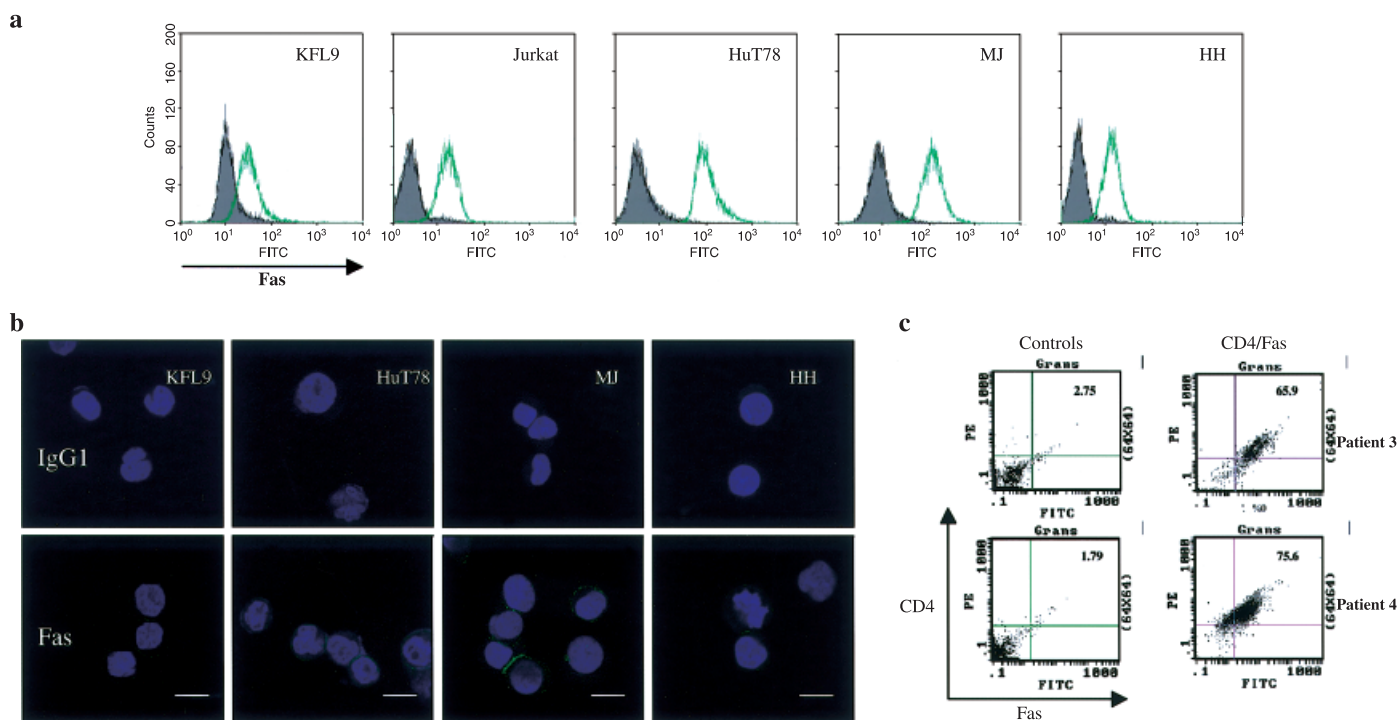


Figure 2

Fas is constitutively expressed on cutaneous T cell lymphoma (CTCL) cells and CD4⁺ peripheral blood lymphocytes (PBL) from patients with Sézary syndrome. (a) Flow cytometric analysis was performed on CTCL cell lines, Jurkat cells, and KFL9 cells labeled with ZB4 monoclonal antibody (mAb) (green lines) in a suspension. An isotype-matching IgG1 was stained as a negative control (gray filled lines). A total of 10,000 events were analyzed for each sample. (b) Confocal microscopic analysis was performed after cytospinning cells on silanated glass slides. Three cells (KFL9 and HH cells) and five cells (HuT78 and MJ cells) were photographed using an Olympus FV500 Confocal Microscope under $\times 60$ magnifications. *Upper panel:* IgG1 staining. *Lower panel:* ZB4 mAb staining. Scale bar: 10 μ m. (c) Flow cytometric analysis was performed on PBL from patients with a high percentage of circulating CD4⁺ CD26[−] cells using ZB4 mAb and CD4 mAb co-labeled. The isotype-matching IgG were used as negative controls. Data are results from patients 3 and 4.

cells. As shown in Fig 5, FasL cell surface expression on HuT78 cells was first detectable at 24 h after activation with CD3 mAb, and continuously increased from 48 to 72 h. Compared with that at baseline (0.3%), cell surface FasL on HuT78 cells was 6.3% at 24 h, 6.6% at 48 h, and 14.7% at 72 h, respectively. FasL surface expression on MJ cells was detectable at 3 h with a low level. But FasL expression on HH cells was detected at 3 h at a high level, which was similar to that on Jurkat cells (Fig 5). These data suggest that different time and amount of FasL expression after activation may be associated with their sensitivity to AICD.

CTCL cells mediate bystander cytotoxicity toward Fas⁺ target cells To further determine the function of FasL ex-

pressed by CTCL cells, we examined CTCL cells' cytolytic activity towards Fas⁺ target cells. We chose Jurkat T cells as target cells because they display high sensitivity toward Fas mAb (Matzinger *et al*, 1991). As shown in Fig 6a, three CTCL cell lines displayed target to effector-dependent cytotoxicity to Jurkat cell targets. HuT78 cells had the highest cytotoxicity to Jurkat cells at each target to effector cell ratio as follows: 9.1% (1:1), 28.1% (1:5), 49.2% (1:10), and 71.6% (1:20), respectively. At ratios 1:10 and 1:20, MJ-induced cell death of 22.4% and 35.2% of Jurkat target cells. HH cells were least cytotoxic, killing only 10.3% of Jurkat cells at the ratio of 1:20 (Fig 6a).

As shown in Fig 6, in Hut78 cells, we were able to block cytotoxicity by pretreating Hut78 cells with neutralizing

Table I. Demographics and immunophenotyping characteristics of Sézary Syndrome patients

| Patient no. | Age/sex | Disease stage | CD3 ⁺ /CD4 ⁺ cells (%) | CD3 ⁺ /CD8 ⁺ T cells (%) | CD4:CD8 ratio | CD4 ⁺ /CD26 [−] T cells (%) | Fas ⁺ /CD4 ⁺ T cells (%) |
|-------------|---------|---------------|--|--|---------------|---|--|
| 1 | 50/M | IVA | 89.9 | 2.8 | 32.1 | 88.7 | N/A |
| | | | 92.1 | 2.3 | 40.0 | 92.1 | 80.1 |
| 2 | 74/M | IVB | 81.6 | 6.2 | 13.2 | 80.3 | 52.3 |
| 3 | 66/F | IVA | 96.0 | 1.6 | 60.0 | 93.3 | 63.2 |
| 4 | 63/M | IVA | 87.3 | 2.3 | 37.9 | 80.7 | 73.8 |

M, male; F, female; N/A, not applicable.

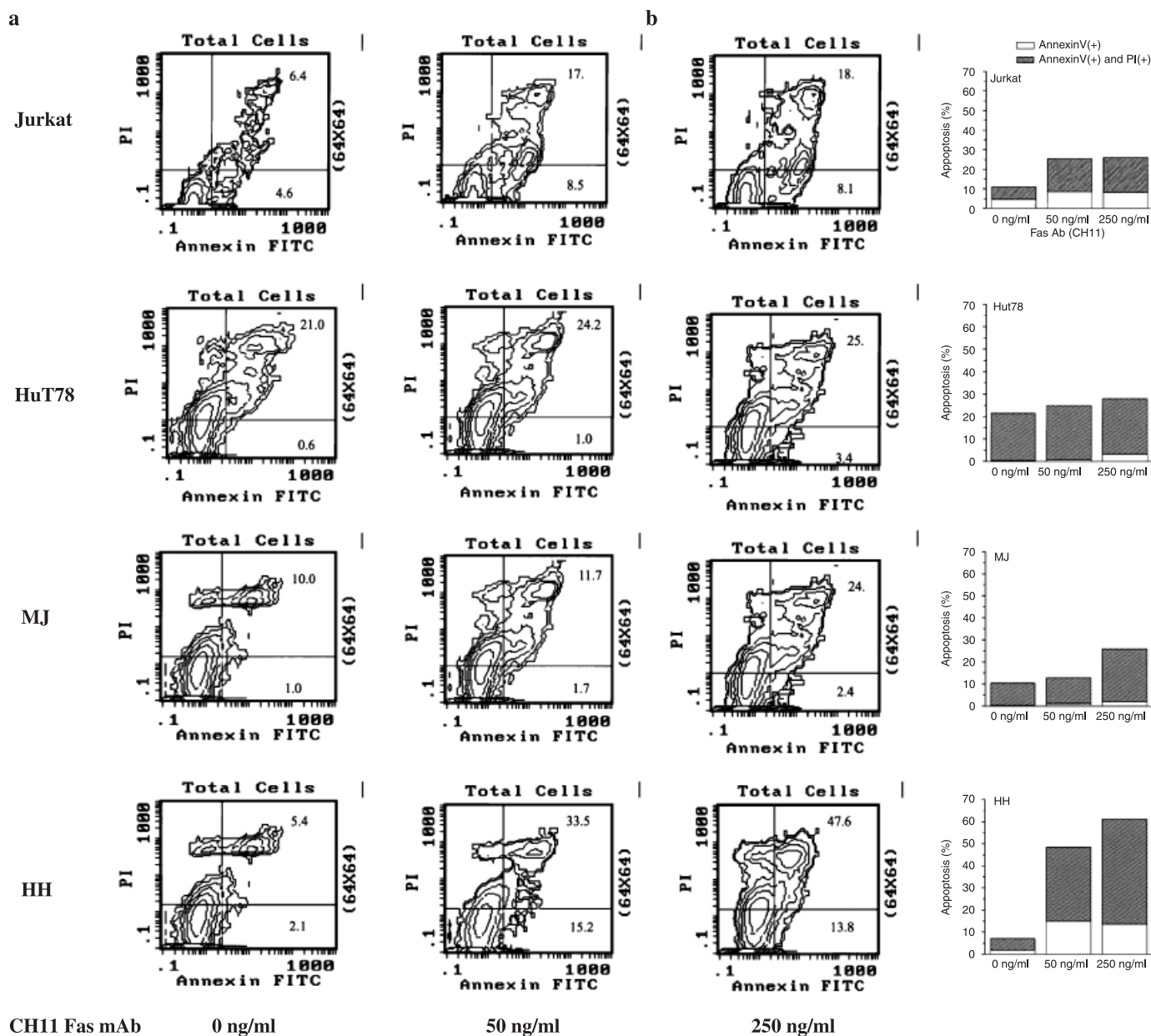


Figure 3

Sensitivity to anti-Fas-inducing apoptosis varies among cutaneous T cell lymphoma cell lines. (a) HuT78, MJ, HH, and Jurkat cells were incubated with different concentrations (0, 50, and 250 ng per mL) of apoptosis-inducing CH11 Fas monoclonal antibody (mAb) for 16 h. Apoptosis was measured by staining with fluorescein isothiocyanate (FITC)-labeled AnnexinV/(propidium iodide (PI)) analyzed with a FACScan (Becton Dickinson). The percentages of early apoptotic cells (stained only AnnexinV) and late apoptotic cells (stained both AnnexinV and PI) are shown in the diagrams. (b) Bar graph of data for each cell line at different mAb dose levels.

antibody to FasL (NOK-2) (Fig 6b) or inhibit the cytotoxicity by pretreating Jurkat cells with antagonistic antibody to Fas (ZB4) (Fig 6c) in a coculture containing Jurkat cells to HuT78 cells of 1:10. These data indicate that FasL expressed by CTCL is functional.

As shown in Fig 6d, target Jurkat cells were cocultured with PBL isolated from patients with SS-PBL and were compared with PBL from the normal donors (N-PBL). SS-PBL from all five samples derived from four different patients had higher cytotoxicity towards target Jurkat cells than did control N-PBL. The cytotoxicity was also increased as the ratio of target cells to effectors increased from 1:1, 1:5, 1:10, to 1:20 (two-sample *t* test with unequal variance

provides 79%, 92%, 95%, and 93% power to detect the differences at a significance level of 0.05 (two sided), respectively).

Discussion

The Fas/FasL pathway may play a dual role in CTCL by allowing the accumulation of neoplastic CD4+ T cells as well as by facilitating the loss of CD8+ cytotoxic T cells. Thus, we were interested in learning how the Fas/FasL machinery functions in CTCL cells. In this study, three human CTCL cell lines and SS-PBL were analyzed for their

Table II. CH11 Fas mAb-induced apoptosis and CD3 mAb-induced AICD in PBL from patients with Sézary syndrome

| Annexin V + /PI + apoptotic cells (%) | Bexarotene (20 μ m per mL) | Fas mAb (CH11) (ng per mL) | | | AICD (2.5 μ g per mL) |
|---------------------------------------|--------------------------------|----------------------------|------|-------|---------------------------|
| | | 50 | 250 | 500 | |
| Patient 1 | 32.6 | 0.5 | 0.7 | 1.8 | 2.9 |
| Patient 2 | 12.3 | -1.4 | -8.5 | -10.1 | 6.3 |
| Patient 3 | 8.8 | 0.3 | -5.0 | -8.0 | -13.5 |
| Patient 4 | 9.1 | 1.3 | 2.9 | 6.6 | -5.6 |

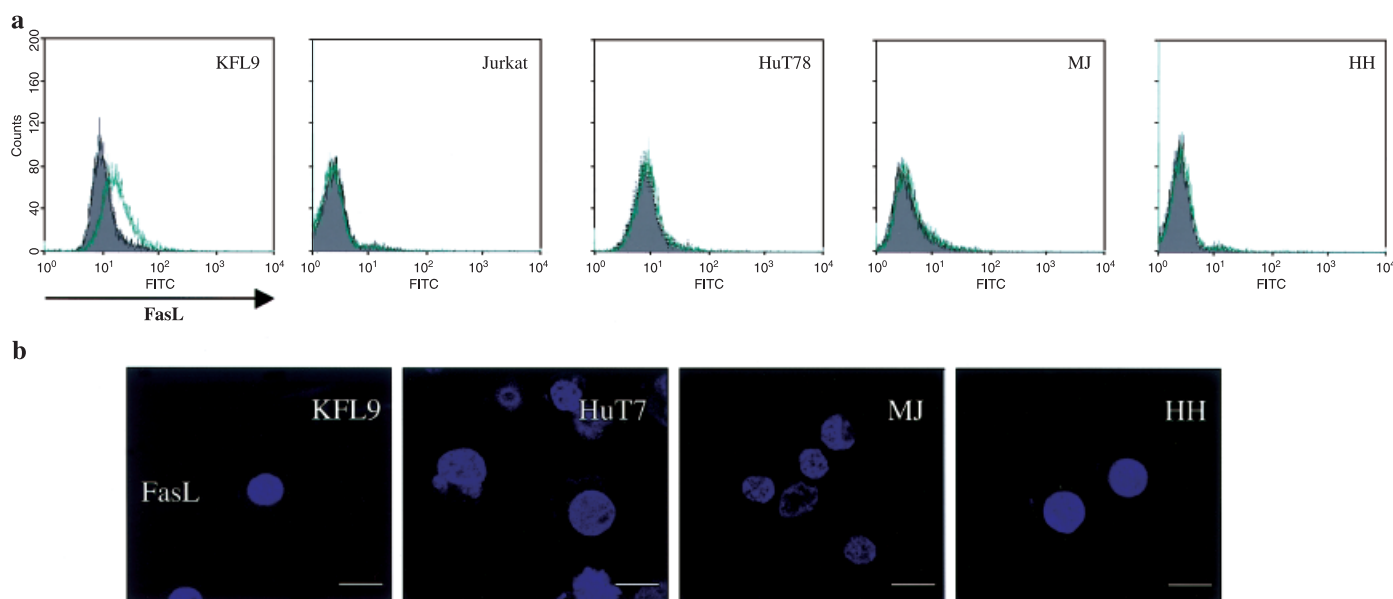
mAb, monoclonal antibody; AICD, activation-induced cell death; PBL, peripheral blood lymphocytes.

responses to Fas mAb treatment and AICD mediated by CD3/TCR ligation. FasL cytotoxic activity of CTCL cells towards Fas⁺ Jurkat target cells was also evaluated and bystander cytotoxicity to Fas⁺ target cells was demonstrated. Differences in susceptibility to Fas mAb and resistance to AICD were found among CTCL cell lines. Surprisingly, resistance to AICD in CTCL cells was not because of lack of expression of Fas on the cells, but perhaps rather to decreased or/and delayed FasL surface expression. These findings describe a possible novel mechanism that neoplastic T cells may utilize to resist AICD and over expand.

Normal naïve CD4⁺ T cells proliferate in response to superantigens or peptides on antigen-presenting cells. When these cells are rested and rechallenged, they undergo AICD mediated by Fas/FasL interactions (Wang *et al*, 1997). Neoplastic T cells from CTCL patients are considered as a population of "genotraumatic" CD4⁺ T cells activated by unknown persistent antigens (Thestrup and Kaltot, 1994; De Panfilis, 2002). If the Fas/FasL-mediated pathway is either lacking or altered in CTCL cells, they would be unable

to undergo AICD and would accumulate causing clinical symptoms and lesions (Dereure *et al*, 2000; Zoi-Toli *et al*, 2000; Ni *et al*, 2001; De Panfilis, 2002). Fas mutations are infrequent in early MF (Dereure, 2002). We found that a comparable expression of Fas is present at all tested CTCL cells, but the sensitivity to CD95 and AICD varies among these cells. This suggests that a normal expression of Fas does not exclude the possibility of a functional impairment of the molecule, i.e., abnormal ligand or through modification of its intracellular part interacting with the connecting molecules involved in cell death triggering.

Expression of FasL on cell surface has been thought to be required during a crucial period after activation in order for T cells to commit to AICD (Nguyen and Russell, 2001). In normal CD4⁺ T cells, FasL appears on the cell surface within minutes after activation, and membrane-bound or cleaved FasL molecules bind to Fas receptors on the surface of the same or on a neighboring cell to trigger the death cascade (Bossi and Griffiths, 1999; De Panfilis, 2002). Our study has showed that CTCL cells are resistant to

**Figure 4**

No Fas ligand (FasL) surface expression on cutaneous T cell lymphoma (CTCL) cells at baseline. (a) Flow cytometric analysis was performed on CTCL cell lines and KFL9 cells labeled with NOK1 mAb (green lines) in a suspension. An isotype-matching IgG1 was stained as a negative control (gray filled lines). (b) Confocal microscopic analysis was performed after cytospinning cells on silanated glass slides. Two cells (KFL9 and HH cells) and five cells (HuT78 and MJ cells) were photographed using an Olympus FV500 Confocal Microscope under $\times 60$ magnifications. Scale bar: 10 μ m.

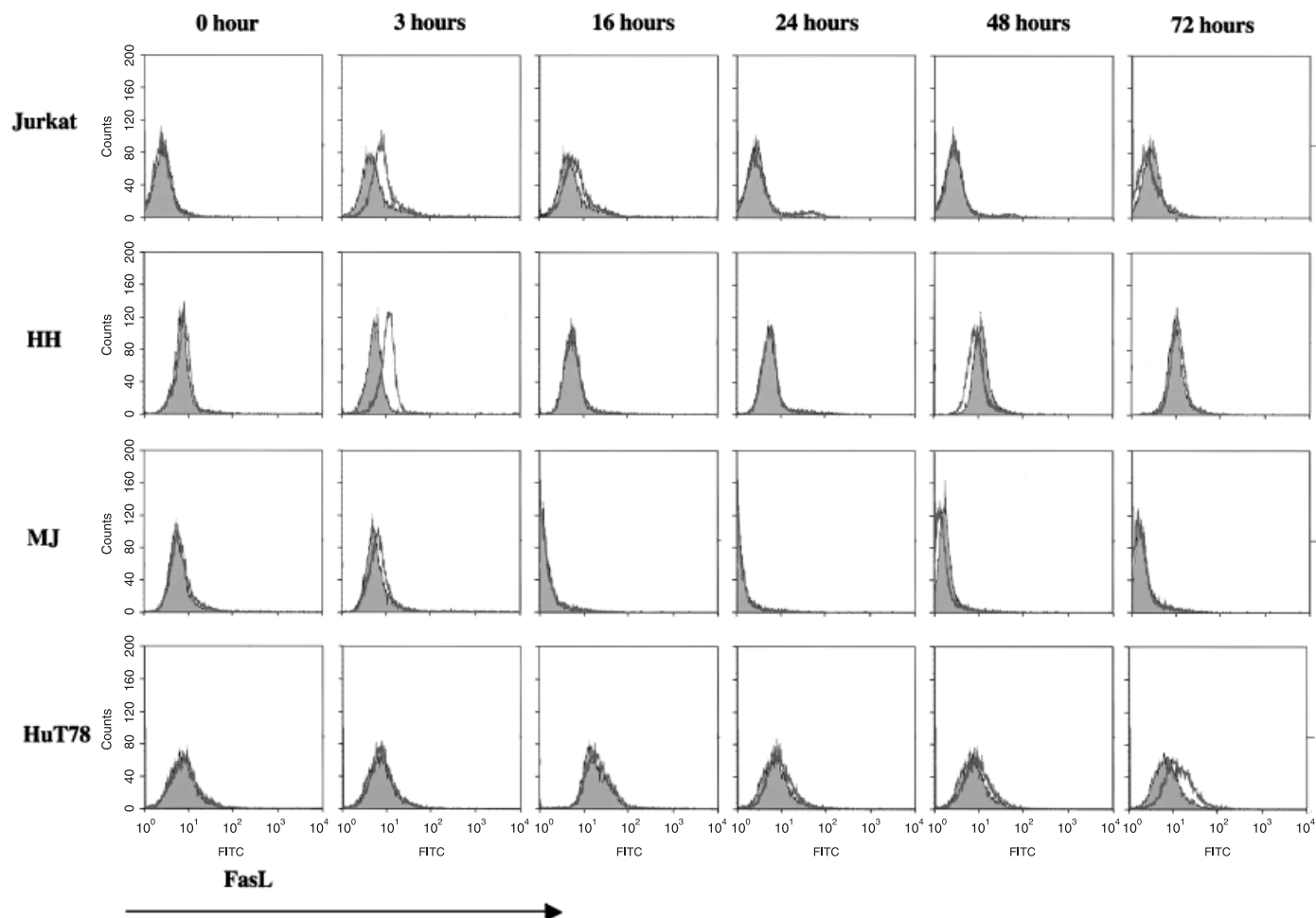


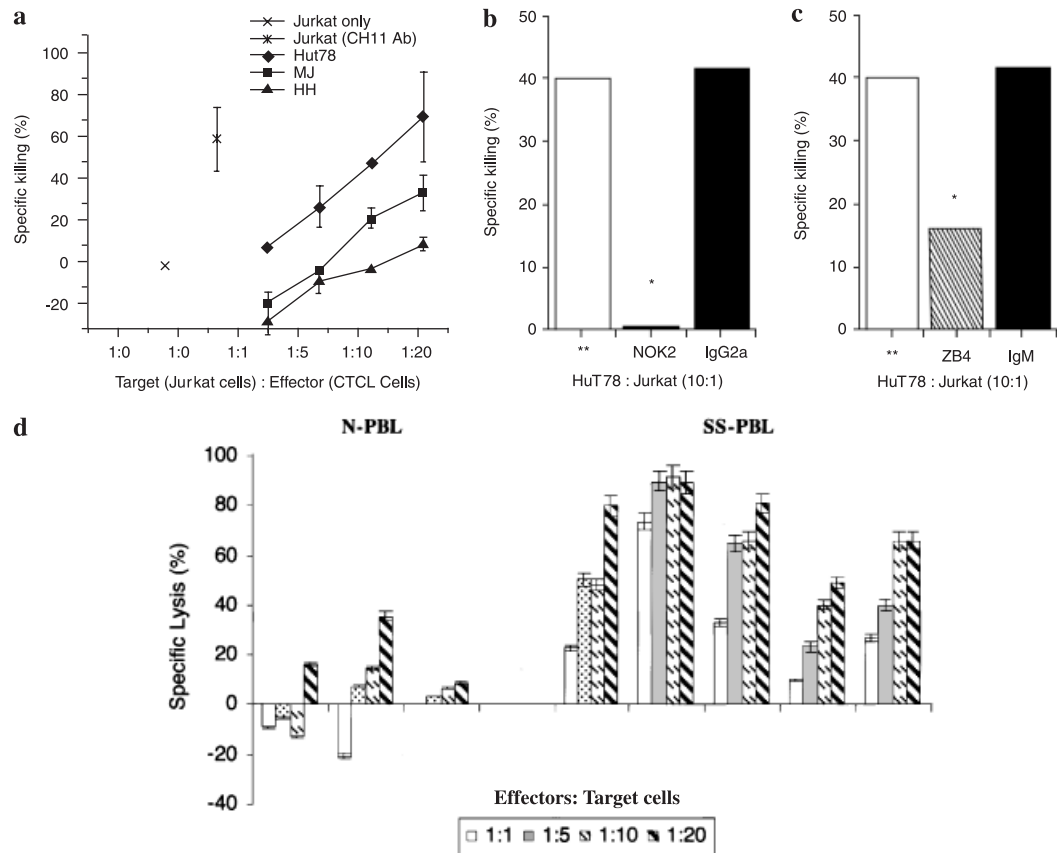
Figure 5
Decreased or/and delayed cell surface Fas ligand (FasL) induction in cutaneous T cell lymphoma (CTCL) cells after CD3 monoclonal antibody (mAb) activation. HuT78, MJ, HH, and Jurkat cells were activated on UTCH-1CD3 mAb-coated plate for different times (3, 16, 24, 48, and 72 h). Cells from baseline and different activation time points were then labeled with NOK-1 mAb (empty lines) in a suspension analyzed with a FACScan. The isotype-matched IgG1 was used as negative control (gray filled lines).

activation-induced apoptosis. In addition, we are first to demonstrate that FasL surface expression is absent at baseline and decreased or/and delayed following activation in those resistant cells, HuT78, and MJ cells. Thus, CTCL cells may resist AICD because of a decreased or/and delayed in the expression of a critical amount of FasL on their cell surface. Others have demonstrated that most FasL expressed by activated T cells is located into cytoplasmic granules, together with perforin and granzyme. The interaction between effector and target cell is associated with polarization of granules toward the cell-cell interface, followed by release of FasL and granules into the intercellular space (Bossi and Griffiths, 1999). Thus, we propose a working hypothesis that by granule release, a CTCL cell may be able to kill a target cell using a FasL-dependent mechanism, in the absence of detectable FasL protein surface expression. Interestingly, a study has found that the lack of interleukin (IL)-2, which is common in SS patients, possibly delayed the expression of FasL so that it was not expressed during the crucial period (Nguyen and Russell, 2001). But the results from the cell lines may or may not reflect the actual behavior *in vivo*; more *ex vivo* or *in vivo* studies need to be carried out in the future to test this hypothesis.

During T cell development, CD4⁺ Th cells differentiate in response to antigen stimulation into Th1 or Th2 cells, defined by their cytokine expression pattern. Th1 cells produce IFN- γ , TNF- α , and IL-2 that are important for the cell-mediated immune response against intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-6, and IL-10, which are involved in humoral immunity (Li-Weber and Krammer, 2002). As mentioned above, after encountering either a superantigen or a specific antigen, the majority of activated T cells are deleted by AICD. Many studies have, however, shown that deletion is never complete (Carbonari *et al*, 2000). Residual undeleted cells after activation express high levels of Th2-type cytokines, indicating that Th2 cells are resistant to AICD (Carbonari *et al*, 2000; Dirsch *et al*, 2001). Although the aberrant cytokine production by Sèzary cells resembles that of murine Th2 cells, the mechanism(s) leading to the dominance of type-2 responses of these cells has not been clarified (Vowels *et al*, 1992, 1994). Our study has demonstrated that in spite of different sensitivity to anti-Fas mAb-induced apoptosis, all three CTCL cell lines were resistant to AICD triggered by CD3 mAb. We hypothesize that AICD resistant, Th2 CTCL cells are able to survive an immune response followed by AICD and to contribute to the pool of

Figure 6

Fas ligand (FasL)-mediated bystander cytotoxicity by cutaneous T cell lymphoma (CTCL) cells. (a) Jurkat cells (2×10^4 cells per mL) labeled with [3 H]-thymidine were co-cultured with HuT78 (\blacklozenge), MJ (\blacksquare), or HH (\blacktriangle) at 2×10^4 , 1×10^5 , 2×10^5 , and 4×10^5 per mL in 96-well plates for 24 h at 37°C. The incorporated radioactivity from undegraded chromosomal DNA of Jurkat cells was measured. Reduction in incorporated radioactivity was used to calculate the percentage of specific target cell killing: ((cpm untreated cells-cpm cocultured cells)/cpm untreated cells \times 100). Treatment of Jurkat cells with 0.25 μ g per mL of death-inducing CH11 mAb served as a positive control (-*-). The negative control for spontaneous cell death was wells containing only Jurkat cells without effector cells (-x-). (b) Specific killing of target cells was blocked when HuT78 cells were incubated with 2.5 μ g per mL of NOK-2 FasL mAb before coculture with labeled Jurkat cells, but not with a relevant negative control IgG2a mAb. (c) The specific killing of Jurkat cells was inhibited when Jurkat cells were incubated with 0.25 μ g per mL of the Fas receptor blocking ZB4 mAb before co-culture with HuT78 cells, but not with a relevant negative control IgM mAb. (d) Jurkat cells labeled with [3 H]-thymidine were co-cultured with peripheral blood lymphocytes (PBL) from patients with high circulating Sézary syndrome (SS) cells (SS-PBL) and normal donors (N-PBL) at the ratios of target cells to effector cells of 1:1; 1:5; 1:10; 1:20. The incorporated radioactivity from Jurkat cell DNA was also measured and the specific target killing (%) was presented.



long-term helper/memory T cells. Other studies have also shown significant differences in the level of FasL surface expression induced in AICD-susceptible Th1 cells, but not in AICD-resistant clone Th2 cells (Oberg *et al*, 1997; Varadhachary *et al*, 1997). The molecular basis for the differential expression of FasL in Th1 and Th2 cells is unknown. Li-Weber and Krammer (2002) have reported that NF-AT and early growth response protein seems to play a major role in FasL expression upon TCR stimulation.

The regulatory mechanism for FasL surface expression is not fully understood. Because Fas is a death-inducing receptor and FasL must be tightly controlled at different levels, these may include delivery to the cell surface, cytokine release, as well as cell membrane-associated matrix metalloproteinases (MMP). FasL is cleaved from the cell surface by several members of the MMP family (Kayagaki *et al*, 1995; Webb *et al*, 2002). Treatment of activated T cells or tumor cell lines with MMP inhibitors *in vitro* leads to the accumulation of surface FasL (Strauss *et al*, 2003). Treatment of all three of CTCL cell lines used in the study with a potent broad-spectrum MMP inhibitor had little effect on the level of FasL on the cell surface (not shown). This result is consistent with Nguyen and Russell's (2001) observation that MMP inhibitors have little effect on the loss of FasL in T cells, but that downregulation of FasL is because of endocytosis.

In CTCL, loss of the cytotoxic CD8⁺ T cells is correlated with disease progression and loss of response to photopheresis (Heald *et al*, 1992). Tumors can escape from immune surveillance by expressing FasL on their surface and thus causing bystander apoptosis of Fas⁺ infiltrating T cells (Green and Ferguson, 2001; Li *et al*, 2002; Abrahams *et al*, 2003). Our work has previously shown that tumor cells in MF skin lesions express FasL, with an inverse correlation between distribution of FasL and CD8⁺ T lymphocytes (Ni *et al*, 2001). This study conducted on cells *in vitro* suggests that CTCL cell lines and SS cells from patients are capable of inducing bystander cytotoxicity of Fas⁺ T target cells. Of interest, HuT78 cells, an SS cell line, showed greatest cytotoxicity and were most resistant to AICD whereas the most sensitive lymphoma cell line HH has the lowest cytotoxic activity against Jurkat cells. In SS, there is a significant increase in circulating activated abnormal CD4⁺ T cells leading to leukemia. FasL-mediated bystander cytotoxic activity by neoplastic CTCL cells could clearly contribute to loss of CD8⁺ T cells seen in SS and MF patients as the disease progresses.

Of interest, all-trans retinoic acid had recently been shown to reduce surface expression of Fas and FasL on acute promyelocytic leukemia cells *in vitro* and to decrease their ability to induce apoptosis of Jurkat T cells (Salih *et al*, 2002). Learning how to best protect the anti-tumor cytotoxic

CD8⁺ T cells in the tumor microenvironment would certainly advance therapeutic strategies for CTCL patients.

Materials and Methods

Established cell lines Three CTCL cell lines (HuT78, MJ, and HH) with an immunophenotype CD3⁺, CD4⁺, and/or CD25⁺ were obtained from American Type Cell Collection (Rockville, Maryland). HuT78 cell line was established from a patient with SS (ATCC number: TIB-161). The MJ cell line was from a patient with MF who was HTLV-1 positive (ATCC number: CRL-8294). The HH cell line was derived from a patient with aggressive cutaneous T cell leukemia/lymphoma (ATCC number: CRL-2105). These cell lines have been shown to express FasL by immunocytochemistry and ELISA (Ni *et al*, 2001). Jurkat T cells (1G5, McKesson HBOC Biosciences, Rockville, Maryland, Cat. Number: 1819) expressing Fas were used as the target in the FasL function assay, and were from Drs E. Aguilar-Cordova and J. Belmont (Baylor College of Medicine, Houston, Texas). The KFL9 cell line, a human FasL transfectant of human myeloid leukemia K562 cells, was a gift from Dr D. Kaplan (Cleveland, Ohio) (Smith *et al*, 1998). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics.

PBL from patients with SS The institutional review board of University of Texas, M. D. Anderson Cancer Center approved all described studies. Experiments performed adhered to Declaration of Helsinki guidelines. Following written consent, venous blood was collected from normal donors and four untreated patients with SS (shown in Table I). These patients had a high percentage of circulating CD4⁺CD26[−] cells that have been shown in two studies to correlate with peripheral Sézary cells (Bernengo *et al*, 2001; Washington *et al*, 2002). From patient 1, two samples with different percentage of CD4⁺CD26[−] T cells were obtained at baseline and 5 mo later. Peripheral blood mononuclear cells were separated by Histopaque-1077 density gradient centrifugation (Sigma, St Louis, Missouri). After removing adhesive monocytes by incubation at 37°C for 1 h, PBL were used for the FasL function assay and immunophenotyping.

Antibodies and reagents For the FasL function assay, we used the following antibodies: mouse anti-human FasL mAb, clone NOK-2 (BD Biosciences, San Diego, California), mouse anti-human Fas mAb, clone CH11 and ZB4 (Kamiya, Thousand Oaks, California), and isotype-matched controls IgG2a and IgM (DakoCytomation, Carpinteria, California). For the FasL and Fas cell surface expression, we used mouse anti-human FasL mAb, clone NOK-1 (BD Biosciences), and mouse anti-human Fas mAb, clone ZB4 as primary antibody, and fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins (DakoCytomation) as secondary antibody. For the characterization of immunophenotype of PBL, phycoerythrin (PE)-conjugated mouse anti-human CD4 mAb, clone RPA-T4, and the isotype control, PE-conjugated IgG1,κ were used (BD Biosciences). For T cell activation, mouse anti-human CD3 mAb, clone UCHT-1 (IgG1,κ, DakoCytomation), PMA, and ionomycin (Sigma) were used. For induction of apoptosis, bexarotene [LGD1069; 4-[1-(3, 5, 5, 8, 8-pentamethyl-5, 6, 7, 8-tetrahydro-2-naphthyl) ethenyl] benzoic acid] (Ligand Pharmaceuticals, San Diego, California) (Zhang *et al*, 2002).

Measurement of AICD CD3 mAb (2.5 μg per mL) was coated to 24-well plates for 1 h at 37°C. The wells were rinsed thoroughly prior to addition of 2 × 10⁵ per well of HuT78, MJ, HH, and Jurkat cells in triplicate. After incubation for 16 h, AnnexinV-FITC/propidium iodide (PI) staining was performed according to the manufacturer's instruction (AnnexinV-FITC Apoptosis Detection Kit, BD Biosciences). Briefly, cells were collected, and suspended in 100 μl Annexin V binding buffer; FITC-conjugated AnnexinV and PI were

added to each sample and incubated for 15 min at room temperature in the dark. After adding 400 μL of binding buffer, the cells were analyzed by flow cytometry. Experiments were repeated twice. The level of AICD (%) was calculated as: ((% death with activation − % death without activation)/(100 − % death without activation).

Measurements of FasL and Fas by flow cytometry analysis

FasL and Fas cell surface expression on CTCL cells KFL9, HuT78, MJ, HH, and Jurkat cells were first incubated with NOK-1 FasL mAb or ZB4 Fas mAb, both at 10 μg per mL on ice for 30 min. FITC-conjugated secondary Ab (1:20) was then added and incubated on ice for 30 min. Following washing, the cells were analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, California). Isotype-matched IgG1 and only secondary Ab were used as negative controls.

FasL and Fas cell surface expression on CD4⁺ PBL PBL from SS patients were first incubated with NOK-1 FasL mAb, or ZB4 Fas mAb followed by FITC-conjugated secondary Ab incubation as described above. Then, PE-conjugated CD4 mAb was incubated on ice for 30 min. The dual-labeled cells were analyzed by flow cytometry. Isotype-matched IgG1 and PE-IgG1,κ were used as the negative controls, respectively.

Measurement of FasL and Fas by confocal microscopic analysis Non-permeabilized HuT78, MJ, HH, and KFL9 cells were incubated with NOK-1 FasL mAb or ZB4 Fas mAb for 45 min on ice, and followed by second anti-mouse FITC-conjugated Ab incubation for 45 min on ice. Cells were then fixed in 1% paraformaldehyde and cytospinned onto silanated glass slides, and analyzed with an Olympus FV500 Confocal Microscope (Olympus American, Melville, New York). An isotype-matched IgG1 was used as a negative staining control.

Fas mAb-induced apoptosis by CH11 antibody CTCL cell lines and Jurkat cells (1 × 10⁶ per mL) were plated in 12-well plates. CH11 Fas mAb was added at concentrations of 0, 50, or 250 ng per mL and incubated for 16 h at 37°C. Annexin V-FITC/PI staining was performed as above. Each data point was performed in triplicate.

FasL-mediated bystander cytotoxicity assay Target Jurkat cells were co-cultured with effector CTCL cells and cytotoxicity was measured by target cell DNA degradation using the JAM test (Matzinger, 1991; O'Connell *et al*, 1996; Villunger *et al*, 1997). Briefly, Jurkat cells incubated with 10 μCi per mL [³H]-thymidine (Amersham, Buckinghamshire, UK) for 5 h were re-suspended at 2 × 10⁴ cells per mL. CTCL cells serially diluted to concentrations of 4 × 10⁵, 2 × 10⁵, 1 × 10⁵, and 2 × 10⁴ per mL in 100 μL aliquots were co-cultured with 100 μL of Jurkat T cell suspensions in 96-well plates for 24 h at 37°C. The final target cell (T) to effector cell (E) ratios were 1:20, 1:10, 1:5, and 1:1. Cells were then harvested and transferred to filter paper. After washing, the incorporated radioactivity from intact chromosomal DNA retained on filters was measured with the β-scintillation counter. Reduction in incorporated radioactivity was used to calculate the percentage of specific target cell killing ((cpm untreated cells − cpm cocultured cells)/cpm untreated cells × 100). For the FasL neutralizing control, CTCL cells were incubated with 2.5 μg per mL of anti-FasL mAb (NOK-2) or with a relevant negative control IgG2a mAb, respectively, 30 min before co-cultivation with labeled Jurkat cells. For the Fas agonist control, labeled Jurkat cells were incubated with 0.25 μg per mL of the Fas receptor blocking ZB4 mAb or a relevant negative control IgM mAb, respectively, 30 min before co-cultivation with CTCL cells. Treatment of labeled Jurkat cells with 0.25 μg per mL of death-inducing anti-Fas mAb, clone CH11, served as a positive control for Fas-mediated apoptosis. The negative controls for spontaneous cell death were wells containing only Jurkat cells without effector cells. The cytotoxicity of Jurkat cells towards CTCL cells was also performed using [³H]-thymidine-labeled CTCL

cells exposed to unlabeled Jurkat cells at the same effector cell/target cell (E/T) ratios. All experiments were performed in triplicate.

Statistics Statistical significance of results was determined by χ^2 test and *t* test. Differences between experimental groups were considered significant at $p < 0.05$.

This work was supported in part by National Institutes of Health Grants (R21-CA74117 and K24-CA 86815), Sherry Anderson Cutaneous T Cell Lymphoma Patient Research and Education Fund, and Dermatology Foundation Research Grant (GCJ7001Z). This paper was presented in part at the Society for Investigative Dermatology Meeting, May 15–18, 2002, Los Angeles, CA. We thank Karen Ramirez and Wendy Schober for expert technical assistance in flow cytometry analysis, and Randall Evans, PhD for confocal microscopic analysis (NIH grant #5P30CA016672-29, Houston, TX). Thanks are also due to Hongbing Shen, PhD and Ying Yang, MS for statistical analysis (Houston, TX), to Tinhofer, PhD, for useful suggestions for cytotoxicity experiment (Innsbruck, Austria), to Francois Martin, MD for helpful discussion (Dijon, France), and to Dorothy Lewis, PhD for critical reading (Houston, TX).

DOI: 10.1111/j.0022-202X.2005.23657.x

Manuscript received July 22, 2004; revised October 29, 2004; accepted for publication November 29, 2004

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